Targeted Enzyme Activated Two-Photon Fluorescent Probes: A Case Study of CYP3A4 Using a Two-Dimensional Design Strategy

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Abstract: Nowadays, the rapid development of fluorescent probe for monitoring target enzyme is still a great challenge, due to the lack of efficient way to optimize a specific fluorophore. Herein, a practical two-dimension molecular strategy was designed to construct an isoform-specific probe for CYP3A4, a key cytochrome P450 isomorph responsible for oxidize most of clinical drugs. In first dimension of the molecular design, using ensemble-based virtual screening, the potential two-photon fluorophore substrate (NN) for CYP3A4 was effectively filtered. For second dimension by chemical modification, various substituent groups were introduced to candidate NN for optimizing the isoform-selectivity and reactivity. Finally, with ideal selectivity and sensitivity, NEN was successfully applied to real-time detect CYP3A4 in living cells and zebrasfish, thereby providing an efficient tool for quantitative tracking of CYP3A4 activity in complex biological systems. These findings suggested that our strategy is practical to develop an isofrm-specific probe for a target enzyme.

Cytochrome P450 monooxygenase (CYP) is a superfamily of oxidative enzymes that metabolizes thousands of endogenous and exogenous substances through alkyl carbon and aromatic ring hydroxylation, O- and N-dealkylation, and epoxidation.[1] CYP3A4 is regarded as the most important CYP isoform in human, due to its high abundance in liver as well as broad substrate spectrum which contributes to that metabolism of more than 50% of clinical drugs.[2,3] Unfortunately, CYP3A4 activity can be modulated by many clinical drugs, thereby frequently causing unfavorable drug-drug interactions (DDI), further leading to altered clinical outcomes or even life-threatening adverse reactions.[4] Additionally, a significantly inter-individual variability of CYP3A4 activity is frequently reported, arising from a number of sources including genetic polymorphism and the response to environmental influences.[5] These factors have greatly limited the understanding of the precise role of CYP3A4 in drug metabolism and DDI, as a result, negatively impacted clinical medication safety and effectiveness.

To accurately characterize CYP3A4 activity, sensitive technologies capable of real-time monitoring CYP3A4 activity are urgently needed. Traditional detection methods mainly rely upon mass spectrometry or high-performance liquid chromatography,[6,7] while they are not compatible with living cell or in vivo applications. Two-photon (TP) fluorescence microscopy, by virtue of its higher sensitivity, real-time spatial high-resolution imaging, and amenability to deep-tissue bioimaging, has shed new light on monitoring target enzyme activity in complex systems.[9,10] However, there is still no TP fluorescent probe developed for real-time and selective imaging of endogenous CYP3A4 activity in living systems.

Previous attempts to develop a fluorescent probe for CYP3A4 were based on a dealkylation mechanism to release a detectable moiety. The O-alkyl derivatives of coumarin, resorufin, and fluorescein were designed and synthesized, but met with limited success in isofrm selectivity.[10] The leading cause for the poor specificity of these probes is that dealkylation usually is a preferential reaction for CYP1A1 and CYP1A2 rather than CYP3A4, especially with polycyclic aromatic compounds.[11] In the light of the extraordinary ability of CYP3A4 to mediate the aromatic hydroxylation,[12] we hypothesized that the high specific probe of CYP3A4 might be obtained by tuning the fluorescence-generation using site specific hydroxylation to 'Switch-ON' the fluorophore. Nowadays, the greatest challenge in designing an isoform-selective and sensitive activity-based fluorescent probe is how to effectively find a specific fluorophore and optimize the structure.[13] A simple ‘lock and key’ method to acquire a rational fluorophore is urgently needed. Herein, we developed a two-dimensional design strategy to achieve an overall “best-fit” molecular tool for selective detection of CYP3A4, which would provide the molecular design strategy from scratch virtual screening of putative two-proton fluorescent substrates (Scheme 1 and Figure S1).[14] For the first design dimension, an in-house docking program FIPSDock was used to perform the constrained ensemble-based hydroxylation of the specific sites on fluorophore. For sensitive fluorescence detection, the introduction of a hydroxyl to fluorophore substrates producing significant fluorescence response, was set as the restrictive reaction and thus was limited to the catalytic site. To ensemble the conformational changes of CYP3A4 upon binding with the different sites of substrates, we obtained twelve crystallographic human CYP3A4 structures from the Protein Data screening against the Bank (PDB code: 1TQN, 1W0F, 1W0G, 2J0D, 2V0M, 3TJS, [1])

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Finally, NN was selected as the fluorophore in the present study, because it possesses CYP3A4 preference and desirable photophysical properties, such as high photostability and significant two-photon absorbability at the near infrared wavelengths. Hydroxylation at C-4 triggers significant change in the fluorescence spectra, which makes NN a desirable OFF–ON two-photon fluorescent probe based on the mechanism of internal charge transfer (ICT). CYP1A1s and isoforms of other subfamily did not participate in the hydroxylation with the disappearance of emission at 558 nm. However, it can still be hydroxylated by CYP3A5, a highly homologous isoform of CYP3A4.

Subsequently, according to the structural features of NN and CYP3A4 catalytic cavity, we introduced various substituent groups to non-reaction site of NN, to adjust the reactive rate and isoform-selectivity (Figure S2), all of which construct our secondary design dimension. Briefly, the nitrogen atom of NN was selected as the modification site to introduce the substituents including steric bulk, hydrophilic and hydrophobic groups. A library of NN derivatives (NN 1-18) were synthesized to evaluate their potential as selective substrates for CYP3A4 by reaction phenotyping-based experimental screening (Figure S2). When introducing the benzene and its analogues to nitrogen atom of NN (NN 11-13), the reactive rates and isoform-selectivity were decreased. Introducing the hydrophobic substituents including alkanes with different lengths, some short-chain alkyl derivatives such as NN 1 (methyl) and NN 2 (ethyl) exhibited good reactivity and high specificity toward CYP3A4, rather than other derivatives with long-chain alkanes (NN 3-9). Additionally, the hydrophilic substituents (NN 15-18) also decreased the reaction rates. N-ethyl-1,8-naphthalimide (NEN, NN 2) displayed a good reactivity and high selectivity toward CYP3A4 over the other isoforms (Figure S3). Subsequently, we explore the feasibility and practicability of this activatable fluorescence method in the complex biological systems, and thereby examine our two-dimension molecular design strategy.

The specificity of NEN for CYP3A4 was firstly investigated. As shown in Figure 2B, CYP3A4 caused remarkable fluorescence enhancement at 558 nm, whereas there was no obvious...
To understand the potential interfering species (Figure S4). To further verify the selectivity of NEN, inhibition assays were conducted in human liver microsome (HLM) using some selective inhibitors of CYP isoforms. The hydroxylation of NEN can be strongly inhibited by CYP3cide and ketoconazole (TKZ), which are selective and potent inhibitors of CYP3A4 (Figure S5).[10] These results indicated that NEN was highly selective for CYP3A4.

The metabolite of NEN was identified as N-ethyl-4-hydroxy-1,8-naphthalimide (NEHN), $\Phi_1 = 0.199$ in PBS:ACN, $\nu/v = 1:1$ by CYP3A4, by comparison of LC retention times, UV and MS spectra with those of a standard compound (Figure S6). NEN showed extremely weak fluorescence over a wide pH range, while NEHN showed strong fluorescence with maxima at 558 nm over pH range of 7-12 (Figure S7). As a result, CYP3A4-mediated 4-hydroxylation of NEN led to discernible changes in the fluorescence emission (Figure 2A). The fluorescence intensity increased linearly when CYP3A4 was progressively added from 0 to 0.1 mg/mL ($R^2 = 0.997, Y = 2.22 \times 10^{-5} \times x + 539.9$) (Figure 2C). To understand the interaction between NEN and CYP3A4, the kinetics was assayed. As shown in Table S2 and Figure S8, NEN 4-hydroxylation in CYP3A4 and HLM followed the classic Michaeli–Menten kinetics, and displayed high affinity and good reactivity. However, the catalytic efficiency of CYP3A5 was very low, with at least 55-fold lower inherent clearance ($V_{int}/K_{int}$) than CYP3A4. The preferred characteristics and ideal kinetic behavior of probe reaction prompting us to use this probe for quantitative measurement of CYP3A4 activity.

Figure 2. Fluorescence response of NEN towards CYP3A4. A) Proposed mechanism of CYP3A4 triggering the fluorescence response of NEN. B) Fluorescence response of NEN (10 μM) following incubation with various human CYPs. C) Fluorescence spectra of NEN (10 μM) following incubation with increasing concentrations of CYP3A4. CYP3A4 dependent fluorescence intensity change at 558 nm is shown as an inset.

Figure 3. A) The catalytic activity of CYP3A4 in 13 individual HLM using NEN as the probe. B) Correlation between reactivity and the CYP3A4 protein content in HLM. The activity for NEN 4-hydroxylation was expressed by the formation rate of NEHN. $V_{int}/K_{int} = 450/558$ nm.
Figure 4. Two-photon confocal fluorescence images of CYP3A4 in human primary hepatocytes. Cells incubated with NEN (a-c); pretreated with rifampin (d-f); TKZ (g-i). (a, d, g) fluorescence images; (b, e, h) bright field images; (c, f, i) overlay images. Excitation: 800 nm, emission window: 520-560 nm.

Figure 5. Fluorescence images of CYP3A4 in living zebrafish. Fish incubated with NEN (A-a, b, c); with NEN in the present of TKZ (A-d, e, f); with NEN in the present of clinical drugs (10 μM) (B); i) TKZ; ii) indinavir, IDV; iii) nilotinib, NT; iv) acetaminophen, APAP; v) omeprazole, OME; vi) montelukast, MK; vii) sulfaphenazole, SZ; viii) clomethiazole, CZ. Magnified views are inserted in the corresponding overlay images.

Excitation: 800 nm, emission window: 520-560 nm. Scale bar: 50 μm.


Selective two-photon CYP3A4 probe: A highly sensitive two-photon fluorescent probe specific for CYP3A4 was designed based on a two-dimension design strategy that combined ensemble-based virtual screening with rationally structure modification. Facilitating the detection of CYP3A4 activity in living cell, tissue and zebrafish, allowing for the evaluation of risks from CYP3A4-mediated drug-drug interactions in the clinic.


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